
Smac-peptides as Therapeutics against Cancer and Autoimmune Diseases

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The present invention relates to the use of the so-called Smac protein and derivatives thereof to cause apoptosis in cancer cells and self-reactive cells of the immune system.

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Cancer constitutes the fourth leading cause of death in Western countries. As the average age in the Western population steadily rises, so do cancer-related deaths indicating that cancer will be one of the most common causes of death in the 21st century. The aggressive cancer cell phenotype is the result of a variety of genetic and epigenetic alterations leading to deregulation of intracellular signaling pathways. Cancer cells commonly fail to undergo so-called "programmed cell death" or "apoptosis", a signaling process that plays a key role in preventing cell tissues from abnormal growth. Thus, apoptosis defects appear to be a major problem in cancer therapy as they confer resistance to many tumors against current treatment protocols, leading to tumor progression.

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In addition to apoptosis defects found in tumors, defects in the ability to eliminate self-reactive cells of the immune system due to apoptosis resistance are considered to play a key role in the pathogenesis of autoimmune diseases. Autoimmune diseases are characterized in that the cells of the immune system produce antibodies against own organs and molecules or directly attack tissues resulting in the destruction of the latter. A failure of those self-reactive cells to undergo apoptosis leads to the manifestation of the disease. Defects in apoptosis regulation have been identified in autoimmune diseases such as Lupus erythematoses disseminatus or rheumatoid arthritis.

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Apoptosis pathways involve diverse groups of molecules. One set of mediators implicated in apoptosis are so-called caspases, cysteine proteases that cleave their substrates specifically at aspartate residues. Caspases convey the apoptotic signal in a proteolytic

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cascade, with caspases cleaving and activating other caspases which subsequently degrade other cellular targets eventually resulting in cellular breakdown. If one or more steps in this cascade is inhibited in tumor cells, these cells fail to accomplish apoptosis and, thus, continue to grow. Caspase activation itself can be triggered by external stimuli affecting certain cell surface receptors, known to the person skilled in the art as so-called death
5 receptors, or by intracellular stress response via the mitochondria leading to the release of mitochondrial proteins. Known death receptors mediating apoptosis include members of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or TRAIL (TNF-related apoptosis inducing ligand) receptors 1 and 2. Stimulation of death
10 receptors with apoptosis-inducing substances leads, among others, to the activation of caspase-8, which in turn activates other caspases and members of another group of apoptosis mediators. This group is called the Bcl-2 family and is thought to regulate the release of the mitochondrial proteins and, thus, link both pathways together, in order to regulate the downstream acting proteolytic caspase cascade.

15 A failure in activating the caspase cascade is caused by the action of so-called Inhibitors of Apoptosis Proteins (IAPs). IAPs bind to early active caspases, thereby preventing the ongoing of the apoptosis process. They are expressed at high levels in many tumors and, by inhibition of caspases, contribute to the resistance of cancers against apoptosis
20 induction.

A major role in activating the caspase cascade is ascribed to a mammalian protein called Smac in humans (or DIABLO in mice). As disclosed, among others, by Du et al. (Cell 102, 2000, 33-42), Smac is a mitochondrial protein of 239 aminoacids possessing a molecular
25 weight of approximately 25000 Dalton (GenBank accession number AAF87716). In the course of an apoptotic response e.g. upon stimulating CD95- or TRAIL death receptors, Smac is released from mitochondria along with other proteins, e.g. cytochrome c. It has been demonstrated earlier that Smac, once released into the cytosol, can bind to IAPs, particularly to the so-called X-linked IAP (XIAP), the most potent inhibitor of caspases.
30 Binding of Smac to XIAP promotes the proteolytic activation of caspases resulting in apoptosis.

Similar to cancer cells in which activation of caspases is inhibited by IAP-dependent mechanisms, failure to eliminate autoreactive T-cells may be due to a blockade in apoptosis signalling. For physiological elimination of activated lymphocytes death receptor systems such as CD95 play a key role. Increased expression of IAPs or members of the Bcl-2 family in activated T-cells prevents the release of Smac from mitochondria and inhibits the function of the latter.

From the foregoing, it becomes evident that impaired release of Smac and other proteins from mitochondria into the cytosol can cause resistance of tumor cells and cells of the immune system to apoptosis. Overexpression of Smac by transfecting the cells with an expression plasmid carrying the Smac gene is one way to overcome the IAP-caused inhibition of caspases, resulting in an enhanced apoptosis rate. This approach was followed by different research groups, which have found that various types of cancer can thus be treated, e.g. melanoma, breast carcinoma or prostate cancer. However, previous studies do not mention or give any hint to treat neuroblastoma or glioblastoma by overexpressing Smac or related proteins.

A direct delivery of proteins into cells is often limited by the poor permeability of the cell membrane. Recently, Carson et al. (Cancer Research 62 (2002) 18-23) have used purified Smac which was microinjected alone or together with cytochrome c into the cytosol of prostate cancer cells which were initially resistant to apoptosis. However, various problems can be encountered when using microinjection for the delivery of biologically active compounds into cells. Problems include low transfer efficiency or complex manipulation, which would preclude their routine use *in vivo*.

The object of the present invention is to provide a form of Smac that is rapidly internalized into tumor cells and cells of the immune system, e.g. T-cells, by cellular uptake.

This object is attained by a Smac protein / carrier entity comprising

(i) a Smac protein, as disclosed by the GenBank accession number AAF87716, or a derivative or fragment thereof,

(ii) a carrier

- 5 and wherein the Smac protein, fragment or derivative thereof and the carrier are linked together enabling the penetration of the Smac/carrier entity through the cell membrane into the cell.

Said entity will be referred to as Smac/carrier entity hereinafter.

- 10 A further object of the invention is the therapy of cancers and autoimmune diseases which, until now, could not be treated using Smac proteins.

In the context of the present invention, the term derivative or fragment of the Smac protein refers to peptides in which one or more aminoacids of the sequence of 239 aminoacids, as disclosed in GenBank number AAF87716, can be substituted by one or more aminoacids different from the original one(s), or peptides the aminoacid sequence of which is either extended, shortened, or both, on either the aminoterminal, or the carboxyterminal or both ends with respect to the original Smac proteins, provided that the function of the Smac protein remains unaffected.

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In a further embodiment, the present invention includes preferably a peptide comprising aminoacids 56 to 70 of Smac. An even more preferred peptide comprises aminoacids 56 to 62 of Smac. Hereinafter, the latter will be referred to as Smac peptide.

- 25 Most preferably, said derivatives or fragments contain the 4 aminoterminal aminoacids 56 to 59 of Smac. This region mediates the interaction of the Smac protein with IAPs.

The carrier, which is preferably a protein, a fragment or derivative thereof, serves as a vehicle the attachment of which to the Smac protein, fragment or derivative thereof enables the penetration of the Smac/carrier entity through the cell membrane into the cell. Appropriate carriers, in particular proteins, are known to the person skilled in the art and include TAT, influenza virus hemagglutinin, the VP22 protein from herpes simplex virus, Antennapedia, fibroblast growth factor, Galparan (transportan), poly-arginine, Pep-1. Other carriers known to a person skilled in the art which do not belong to proteins, but mediate the internalization of molecules into cells include lipids and cationic lipids.

When a protein is used as a carrier, the term derivative or fragment of a protein refers to peptides in which one or more aminoacids can be substituted by other aminoacids different from the original one(s), or peptides the aminoacid sequence of which is either extended, shortened, or both, on either the aminoterminal, or the carboxyterminal or both ends, with respect to the original one(s), provided that the function as a carrier for the cellular uptake of Smac remains unaffected. The above definition relates to TAT, influenza virus hemagglutinin, the VP22 protein from herpes simplex virus, Antennapedia, fibroblast growth factor, Galparan (transportan), poly-arginine and Pep-1.

The term "carrier" does not include compounds or proteins/peptides linked to or associated with the protein of interest Smac to increase the stability of the Smac/carrier entity, like e.g. alpha-esters, thioamides, sulfonamides, N-hydroxyamides. In addition, the term "carrier" does not include compounds or proteins/peptides, which are linked to the protein of interest (Smac) in order to localize the fusion protein within or purify the fusion protein from cells/cell extracts. Examples for such peptides or compounds are FLAG-tag, myc-tag, HIS-tag, GST-tag, fluorescent dyes, enzymes such as luciferase.

The Smac protein, fragment or derivative thereof is linked to the carrier. This can occur by any chemical interaction known to the person skilled in the art, like coordinative bonds, chemical adsorption, dipole-dipole interaction or the like. Preferably, the carrier is linked to the Smac protein by a chemical bond, in particular a covalent bond, in case the carrier is

a protein. This bond must be such that it remains unaffected before and while penetrating the cell membrane and, if necessary for the interaction of the Smac protein with IAPs, can be cleaved. In general, the Smac/carrier entity can interact with IAPs to the necessary extent, a cleavage being not necessary.

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In a preferred embodiment of the present invention, the carrier is TAT or a derivative or a fragment thereof. TAT is the human immunodeficiency virus-1 (HIV-1) trans-activating protein consisting of 86 aminoacids. More preferably, the fragment or derivative of TAT comprises the aminoacids 37 to 72 of TAT, as disclosed in GenBank accession number
10 CAA45721 (see also M15654 for the complete HIV sequence). It is even more preferred to use, as a carrier, the so-called protein transduction domain of TAT (PTD) which comprises a region on the protein extending from aminoacid residues 47 to 57, according to the disclosed sequence. In this preferred embodiment of the invention, PTD is linked to Smac, or a fragment or derivative thereof.

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The Smac/carrier entity as disclosed in the present invention can be used as a pharmaceutical, optionally in combination with radiation therapy and/or at least one active compound. This is a further embodiment of the present invention.

20 First, the phrase "radiation therapy" refers to the use of electromagnetic or particulate radiation in the treatment of neoplasia. Radiation therapy is based on the principle that high-dose radiation delivered to a target area will result in the death of reproductive cells in both tumor and normal tissues. The radiation dosage regimen is generally defined in terms of radiation absorbed dose (rad), time and fractionation, and must be carefully defined by
25 the oncologist. The amount of radiation a patient receives will depend on various considerations but the two most important considerations are the location of the tumor in relation to other critical structures or organs of the body, and the extent to which the tumor has spread. Examples of radiotherapeutic agents are provided in, but not limited to, radiation therapy and is known in the art. Recent advances in radiation therapy include
30 three-dimensional conformal external beam radiation, intensity modulated radiation therapy (IMRT), stereotactic radiosurgery and brachytherapy (interstitial radiation

therapy), the latter placing the source of radiation directly into the tumor as implanted "seeds". These newer treatment modalities deliver greater doses of radiation to the tumor, which accounts for their increased effectiveness when compared to standard external beam radiation therapy.

5 Ionizing radiation with beta-emitting radionuclides is considered the most useful for radiotherapeutic applications because of the moderate linear energy transfer (LET) of the ionizing particle (electron) and its intermediate range (typically several millimeters in tissue). Gamma rays deliver dosage at lower levels over much greater distances. Alpha
10 particles represent the other extreme; they deliver very high LET dosage, but have an extremely limited range and must, therefore, be in intimate contact with the cells of the tissue to be treated. In addition, alpha emitters are generally heavy metals, which limits the possible chemistry and presents undue hazards from leakage of radionuclide from the area to be treated. Depending on the tumor to be treated all kinds of emitters are conceivable
15 within the scope of the present invention, however, gamma irradiation may be preferred for the purposes of the present invention. Furthermore, the present invention encompasses types of non-ionizing radiation like e.g. ultraviolet (UV) radiation, high energy visible light, microwave radiation (hyperthermia therapy), infrared (IR) radiation and lasers.

20 Generally, radiation therapy can be combined temporally with other active compounds listed below to improve the outcome of treatment. There are various terms to describe the temporal relationship of administering radiation therapy together with other active compounds, and the following examples are the preferred treatment regimens and are generally known by those skilled in the art and are provided for illustration only and are
25 not intended to limit the use of other combinations. Administration of radiation therapy with other active compounds can be "sequential", i.e. separately in time in order to allow the separate administration, "concomitant" which refers to the administration on the same day, and, finally, "alternating" which refers to the administration of radiation therapy on the days in which other active compounds would not have been administered.

30 The term "active compound" refers to a compound other than Smac, a fragment or derivative thereof, which is able to induce apoptosis or which inhibits cell proliferation.

Active compounds which are able to induce apoptosis are known to the person skilled in the art. One class of active compounds are chemical compounds having a cytostatic or antineoplastic effect ("cytostatic compound"). Cytostatic compounds included in the present invention comprise, but are not restricted to (i) antimetabolites, such as cytarabine, fludarabine, 5-fluoro-2'-deoxyuridine, gemcitabine, hydroxyurea or methotrexate; (ii) DNA-fragmenting agents, such as bleomycin, (iii) DNA-crosslinking agents, such as chlorambucil, cisplatin, cyclophosphamide or nitrogen mustard; (iv) intercalating agents such as adriamycin (doxorubicin) or mitoxantrone; (v) protein synthesis inhibitors, such as L-asparaginase, cycloheximide, puromycin or diphtheria toxin; (vi) topoisomerase I poisons, such as camptothecin or topotecan; (vii) topoisomerase II poisons, such as etoposide (VP-16) or teniposide; (viii) microtubule-directed agents, such as colcemid, colchicine, paclitaxel, vinblastine or vincristine; (ix) kinase inhibitors such as flavopiridol, staurosporin, STI571 (CPG 57148B) or UCN-01 (7-hydroxystaurosporine); (x) miscellaneous investigational agents such as PS-341, phenylbutyrate, ET-18-OCH₃, or farnesyl transferase inhibitors (L-739749, L-744832); polyphenols such as quercetin, resveratrol, piceatannol, epigallocatechine gallate, theaflavins, flavanols, procyanidins, betulinic acid and derivatives thereof; (xi) hormones such as glucocorticoids or fenretinide; (xii) hormone antagonists, such as tamoxifen, finasteride or LHRH antagonists.

Other cytostatic compounds, which are included in the present invention, include plant-derived cytostatics (from *Viscum* and derivatives); alkaloids such as vindesine; podophyllotoxins such as vinorelbin; alkylants such as nimustine, carmustine, lomustine, estramustine, melphalam, ifosfamide, trofosfamide, bendamustine, dacarbazine, busulfane, procarbazine, treosulfane, tremozolamide, thiotepa; cytotoxic antibiotics such as aclarubicine, daunorubicine, epirubicine, idarubicine, mitomycine, dactinomycine; antimetabolites like folic acid analogs such as methotrexate, purine analogs such as cladribin, mercaptopurin, tioguanine and pyrimidine analogs such as cytarabine, fluorouracil, docetaxel; platinum compounds such as thioplatin, carboplatin, oxaliplatin; amsacrine, irinotecan, interferon- α , tretinoine, hydroxycarbamide, miltefosine, pentostatine, aldesleukine; antineoplastic compounds derived from organs, e.g. monoclonal antibodies such as trastuzumab, rituximab, or derived from enzymes such as pegaspargase;

endocrine effecting antineoplastic compounds belonging to hormones, e.g. estrogens such as polyestradiol, fosfestriol, ethinylestradiol, gestagens such as medroxyprogesterone, gestonoroncaproat, megestrol, norethisterone, lynestrenol, hypothalamus hormones such as triptoreline, leuproreline, busereline, gosereline, other hormones such as testolactone, testosterone; endocrine effecting antineoplastic compounds belonging to hormone antagonists, e.g. antiestrogens such as toremifen; antiandrogens such as flutamide, bicalutamide, cyproterane; endocrine effecting antineoplastic compounds belonging to enzyme inhibitors such as anastrol, exemestane, letrozol, formestane, aminoglutethimide, all of which can be occasionally administered together with so-called protectives such as calciumfolinat, amifostin, lenograstin, molgromostin, filgrastin, mesna or so-called additives such as retinolpalmitate, thymus D9, amilomer.

Another class of active compounds which can be used in the present invention are those which are able to induce apoptosis by binding to death receptors ("death receptor ligands"). They include tumor necrosis factor α (TNF- α), tumor necrosis factor β (TNF- β , lymphotoxin- α), LT- β (lymphotoxin- β), TRAIL (Apo2L), CD95 (Fas, APO-1) ligand, TRAMP (DR3, Apo-3) ligand, DR4 ligand, DR6 ligand as well as fragments and derivatives of any of said ligands. Preferably, the death receptor ligand is selected from the group consisting of TNF- α , a fragment or derivative thereof, and TRAIL, a fragment and derivative thereof.

Other active compounds include agonistic antibodies to death receptors such as anti-CD95 antibody, anti-TRAIL-R1 (DR4) antibody, anti-TRAIL-R2 (DR5) antibody, anti-DR6 antibody, anti TNF-R1 antibody and anti-TRAMP (DR3) antibody as well as fragments and derivatives of any of said antibodies. Preferably, the agonistic antibodies are selected from the group consisting of anti-TRAIL-R1 antibody, anti-TRAIL-R2 antibody, anti TNF-R1 antibody and fragments and derivatives of any of said antibodies.

The preferred Smac/carrier entity of the present invention is the Smac peptide linked to PTD, and will be referred to as Smac peptide/PTD hereafter.

In the present invention, the cytostatic compound used in combination with the Smac/carrier entity is preferably selected from the group consisting of doxorubicin, cisplatin and etoposide (VP-16). Further preferred active compounds of the present invention used in combination with the Smac/carrier entity are selected from the group of death receptor agonists consisting of TRAIL, anti-CD95 antibody and derivatives and fragments of any of said agonists.

The Smac/carrier entity can be administered alone or in combination with one or more active compounds. The latter can be administered before, after or simultaneously with the administration of the Smac/carrier entity. The dose of either the Smac/carrier entity or the active compound as well as the duration and the temperature of incubation can be variable and depends on the target that is to be treated.

A further object of the present invention are pharmaceutical preparations which comprise an effective dose of at least one Smac/carrier entity and/or at least one active compound and a pharmaceutically acceptable carrier, i.e. one or more pharmaceutically acceptable carrier substances and/or additives.

The pharmaceutical according to the invention can be administered orally, for example in the form of pills, tablets, lacquered tablets, sugar-coated tablets, granules, hard and soft gelatin capsules, aqueous, alcoholic or oily solutions, syrups, emulsions or suspensions, or rectally, for example in the form of suppositories. Administration can also be carried out parenterally, for example subcutaneously, intramuscularly or intravenously in the form of solutions for injection or infusion. Other suitable administration forms are, for example, percutaneous or topical administration, for example in the form of ointments, tinctures, sprays or transdermal therapeutic systems, or the inhalative administration in the form of nasal sprays or aerosol mixtures, or, for example, microcapsules, implants or rods. The preferred administration form depends, for example, on the disease to be treated and on its severity.

The preparation of the pharmaceutical compositions can be carried out in a manner known per se. To this end, the Smac/carrier entity and/or the active compound, together with one or more solid or liquid pharmaceutical carrier substances and/or additives (or auxiliary substances) and, if desired, in combination with other pharmaceutically active compounds having therapeutic or prophylactic action, are brought into a suitable administration form or dosage form which can then be used as a pharmaceutical in human or veterinary medicine.

For the production of pills, tablets, sugar-coated tablets and hard gelatin capsules it is possible to use, for example, lactose, starch, for example maize starch, or starch derivatives, talc, stearic acid or its salts, etc. Carriers for soft gelatin capsules and suppositories are, for example, fats, waxes, semisolid and liquid polyols, natural or hardened oils, etc. Suitable carriers for the preparation of solutions, for example of solutions for injection, or of emulsions or syrups are, for example, water, physiological sodium chloride solution, alcohols such as ethanol, glycerol, polyols, sucrose, invert sugar, glucose, mannitol, vegetable oils, etc. It is also possible to lyophilize the Smac/carrier entity and/or the active compound and to use the resulting lyophilisates, for example, for preparing preparations for injection or infusion. Suitable carriers for microcapsules, implants or rods are, for example, copolymers of glycolic acid and lactic acid.

The pharmaceutical preparations can also contain additives, for example fillers, disintegrants, binders, lubricants, wetting agents, stabilizers, emulsifiers, dispersants, preservatives, sweeteners, colorants, flavorings, aromatizers, thickeners, diluents, buffer substances, solvents, solubilizers, agents for achieving a depot effect, salts for altering the osmotic pressure, coating agents or antioxidants.

The dosage of the Smac/carrier entity, in combination with one or more active compounds to be administered, depends on the individual case and is, as is customary, to be adapted to the individual circumstances to achieve an optimum effect. Thus, it depends on the nature

and the severity of the disorder to be treated, and also on the sex, age, weight and individual responsiveness of the human or animal to be treated, on the efficacy and duration of action of the compounds used, on whether the therapy is acute or chronic or prophylactic, or on whether other active compounds are administered in addition to the Smac/carrier entity.

The Smac/carrier entities according to the present invention, respectively the medicaments containing the latter, can be used for the treatment of all cancer types which are resistant to apoptosis due to the expression of IAPs. Examples of such cancer types comprise neuroblastoma, intestinal carcinoma such as rectum carcinoma, colon carcinoma, familial adenomatous polyposis carcinoma and hereditary non-polyposis colorectal cancer, esophageal carcinoma, labial carcinoma, larynx carcinoma, hypopharynx carcinoma, tongue carcinoma, salivary gland carcinoma, gastric carcinoma, adenocarcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, renal carcinoma, kidney parenchyma carcinoma, ovarian carcinoma, cervix carcinoma, uterine corpus carcinoma, endometrium carcinoma, chorion carcinoma, pancreatic carcinoma, prostate carcinoma, testis carcinoma, breast carcinoma, urinary carcinoma, melanoma, brain tumors such as glioblastoma, astrocytoma, meningioma, medulloblastoma and peripheral neuroectodermal tumors, Hodgkin lymphoma, non-Hodgkin lymphoma, Burkitt lymphoma, acute lymphatic leukemia (ALL), chronic lymphatic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), adult T-cell leukemia lymphoma, hepatocellular carcinoma, gall bladder carcinoma, bronchial carcinoma, small cell lung carcinoma, non-small cell lung carcinoma, multiple myeloma, basalioma, teratoma, retinoblastoma, chorioidea melanoma, seminoma, rhabdomyosarcoma, craniopharyngeoma, osteosarcoma, chondrosarcoma, myosarcoma, liposarcoma, fibrosarcoma, Ewing sarcoma and plasmocytoma.

Examples of cancer types where the use of the Smac/carrier entities according to the present invention, respectively the medicaments containing the latter, is particularly advantageous include neuroblastoma, glioblastoma, breast carcinoma, melanoma, prostate

carcinoma, pancreatic carcinoma, hepatocellular carcinoma, colon carcinoma, small cell and non-small cell lung carcinoma.

The Smac/carrier entities according to the present invention, respectively the medicaments containing the latter, can furthermore be used for the treatment of all autoimmune diseases which are resistant to apoptosis due to the expression of IAPs or members of the Bcl-2 family. Examples of such autoimmune diseases are collagen diseases such as rheumatoid arthritis, Lupus erythematoses disseminatus, Sharp syndrome, CREST syndrome (calcinosis, Raynaud syndrome, esophageal dysmotility, telangiectasia), dermatomyositis, vasculitis (Morbus Wegener) and Sjögren syndrome, renal diseases such as Goodpasture syndrome, rapidly-progressing glomerulonephritis and membrane-proliferative glomerulonephritis type II, endocrine diseases such as type-I diabetes, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), autoimmune parathyroidism, pernicious anemia, gonad insufficiency, idiopathic Morbus Addison, hyperthyreosis, Hashimoto thyroiditis and primary myxedema, skin diseases such as Pemphigus vulgaris, bullous pemphigoid, Herpes gestationis, Epidermolysis bullosa and Erythema multiforme major, liver diseases such as primary biliary cirrhosis, autoimmune cholangitis, autoimmune hepatitis type-1, autoimmune hepatitis type-2, primary sclerosing cholangitis, neuronal diseases such as multiple sclerosis, Myasthenia gravis, myasthenic Lambert-Eaton syndrome, acquired neuromyotony, Guillain-Barré syndrome (Müller-Fischer syndrome), Stiff-man syndrome, cerebellar degeneration, ataxia, opsoklonus, sensoric neuropathy and achalasia, blood diseases such as autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura (Morbus Werlhof), infectious diseases with associated autoimmune reactions such as AIDS, Malaria and Chagas disease.

In a further embodiment of the present invention neuroblastoma and glioblastoma cells or self-reactive cells of the immune system are treated by administering an active compound in combination with the overexpression of Smac in the cells. The latter is achieved by methods known to persons skilled in the art, preferably by transfecting the cells with an expression plasmid carrying the full length Smac gene, as disclosed in GenBank number AF262240, or a derivative or a fragment thereof.

Active compounds which can be used in the above treatment include cytostatic compounds from the group of antimetabolites, such as cytarabine, fludarabine, 5-fluoro-2'-deoxyuridine, gemcitabine, hydroxyurea or methotrexate; DNA-fragmenting agents, such as bleomycin, DNA-crosslinking agents, such as chlorambucil, cisplatin, cyclophosphamide or nitrogen mustard; intercalating agents such as adriamycin (doxorubicin) or mitoxantrone; protein synthesis inhibitors, such as L-asparaginase, cycloheximide, puromycin or diphtheria toxin; topoisomerase I poisons, such as camptothecin or topotecan; topoisomerase II poisons, such as etoposide (VP-16) or teniposide; microtubule-directed agents, such as colcemid, colchicine, paclitaxel, vinblastine or vincristine; kinase inhibitors such as flavopiridol, staurosporin, STI571 (CPG 57148B) or UCN-01 (7-hydroxystaurosporine); miscellaneous investigational agents such as PS-341, phenylbutyrate, ET-18-OCH₃, or farnesyl transferase inhibitors (L-739749, L-744832); polyphenols such as quercetin, resveratrol, piceatannol, epigallocatechine gallate, theaflavins, flavanols, procyanidins, betulinic acid and derivatives thereof; hormones such as glucocorticoids or fenretinide; hormone antagonists, such as tamoxifen, finasteride or LHRH antagonists; plant-derived cytostatics (from *Viscum* and derivatives); alkaloids such as vindesine; podophyllotoxins such as vinorelbin; alkylants such as nimustrine, carmustine, lomustine, estramustine, melphalam, ifosfamide, trofosfamide, bendamustine, dacarbazine, busulfane, procarbazine, treosulfane, tremozolamide, thiotepe; cytotoxic antibiotics such as aclarubicine, daunorubicine, epirubicine, idarubicine, mitomycin, dactinomycin; antimetabolites like folic acid analogs such as methotrexate, purine analogs such as cladribin, mercaptopurin, tioguanine and pyrimidine analogs such as cytarabine, fluorouracil, docetaxel; other antineoplastic, platinum compounds such as thioplatin, carboplatin, oxaliplatin; amsacrine, irinotecan, interferon- α , tretinoin, hydroxycarbamide, miltefosine, pentostatin, aldesleukine; antineoplastic compounds derived from organs, e.g. monoclonal antibodies such as trastuzumab, rituximab, or derived from enzymes such as pegaspargase; endocrine effecting antineoplastic compounds belonging to hormones, e.g. estrogens such as polyestradiol, fosfestriol, ethinylestradiol, gestagens such as medroxyprogesterone, gestonoroncaproat, megestrol, norethisterone, lynestrenol, hypothalamus hormones such as triptoreline, leuproreline, busereline, gosereline, other hormones such as testolactone,

testosterone; endocrine effecting antineoplastic compounds belonging to hormone antagonists, e.g. antiestrogens such as toremifen; antiandrogens such as flutamide, bicalutamide, cyproterane; endocrine effecting antineoplastic compounds belonging to enzyme inhibitors such as anastrol, exemestane, letrozol, formestane, aminoglutethimide, all of which can be occasionally administered together with so-called protectives such as calciumfolinat, amifostin, lenograstin, molgromostin, filgrastin, mesna or so-called additives such as retinolpalmitate, thymus D9, amilomer.

Preferred active compounds are selected from the group consisting of cisplatin, doxorubicin, and VP-16.

Other active compounds, which can be used for the treatment of tumor cells and self-reactive cells of the immune system overexpressing Smac include death receptor ligands, such as tumor necrosis factor α (TNF- α), tumor necrosis factor β (TNF- β , lymphotoxin- α), LT- β (lymphotoxin- β), TRAIL (Apo2L), CD95 (Fas, APO-1) ligand, TRAMP (DR3, Apo-3) ligand, DR4 ligand, DR6 ligand as well as fragments and derivatives of any of said ligands. Preferably, the death receptor ligand is selected from the group consisting of TNF- α , a fragment or derivative thereof, and TRAIL, a fragment and derivative thereof.

For the treatment of tumor cells overexpressing Smac there can also be used agonistic antibodies to death receptors such as anti-CD95 antibody, anti-TRAIL-R1 (DR4) antibody, anti-TRAIL-R2 (DR5) antibody, anti-DR6 antibody, anti TNF-R1 antibody and anti-TRAMP (DR3) antibody as well as fragments and derivatives of any of said antibodies. Preferably, the agonistic antibodies are selected from the group consisting of anti-TRAIL-R1 antibody, anti-TRAIL-R2 antibody, anti TNF-R1 antibody and fragments and derivatives of any of said antibodies.

The term derivative or fragment of the Smac gene refers to DNA sequences in which one or more nucleotides of the coding sequence of 1358 nucleotides, as disclosed in GenBank

number AF262240, can be substituted by one or more nucleotides different from the original one(s), or Smac DNA sequences the nucleotide sequence of which is either extended, shortened, or both, on either the 5'-, or the 3'- or both ends, provided that the function of the encoded Smac protein remains unaffected.

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A preferred fragment of the Smac gene in the present invention to be overexpressed in tumor cells include the Smac cDNA lacking the nucleotides 20-184 of the disclosed coding sequence, which codes for the so-called mitochondrial targeting sequence (aminoacids 1-55 of the corresponding Smac protein), thus enabling the overexpression of Smac directly in the cytosol, which is the preferred site of Smac action.

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By the administration of an active compound combined with the overexpression of Smac in the cells to be treated, as described beforehand, neuroblastoma and glioblastoma and related types of cancer, like colon carcinoma, hepatocellular carcinoma or small cell and non-small cell lung carcinoma, can be treated successfully. Thus, a further object of the present invention are kits comprising at least one active compound, as described above, and expression plasmids carrying the full length Smac gene, as disclosed in GenBank number AF262240, or a derivative or fragment thereof. The said kits can be used as a medicament for the treatment of neuroblastoma, glioblastoma and related cancers.

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DESCRIPTION OF THE DRAWING

Figure 1. Effect of overexpression of mitochondrial or cytosolic Smac on gamma irradiation-induced apoptosis. SHEP neuroblastoma cells transfected with vector control (A; white bars), mitochondrial Smac (B; hatched bars) or cytosolic Smac (C; black bars) were treated with 0.3-10 Gy gamma irradiation. Apoptosis was determined after 10 days by FACS analysis of propidium iodide-stained DNA. Mean and standard deviation of triplicates of a representative experiment are shown. X-axis represents irradiation dosage (Gy), Y-axis represents percentages (%) of apoptosis.

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EXAMPLES

Overexpression of Smac sensitizes for death receptor or drug-induced apoptosis. A full length Smac construct was used to transfect SHEP neuroblastoma cells, which exhibit intermediate sensitivity to various pro-apoptotic stimuli. Representative experiments performed with clone #28 which overexpressed high levels of Smac are subsequently done. Overexpression of Smac potentiated TRAIL-induced apoptosis in a dose- and time-dependent manner compared to vector control cells and also markedly increased apoptosis induced by anti-CD95 antibody or cytotoxic drugs. Because overexpression of Smac enhanced both death receptor and drug-induced apoptosis, Smac acts at a common point where these two pathways converge, e.g. at the level of postmitochondrial activation of caspases.

Smac sensitizes for apoptosis by antagonizing XIAP. It was investigated whether the apoptosis promoting effect of Smac was mediated by antagonizing XIAP, a prominent caspase inhibitor. Treatment with TRAIL resulted in enhanced release of Smac from mitochondria into the cytosol in cells transfected with Smac compared to vector control cells. Immunoprecipitation of Flag-tagged Smac showed binding of Smac to XIAP upon treatment with TRAIL. Also, immunoprecipitation of endogenous XIAP revealed enhanced binding of Smac to XIAP in Smac transfected cells upon TRAIL treatment compared to vector control cells resulting in complete dissociation of XIAP from caspase-9. Furthermore, overexpression of Smac enhanced activation of caspase-8, -9, -3, cleavage of the caspase substrates PARP and DFF45 and cleavage of Bid and XIAP upon treatment with TRAIL or doxorubicin. These findings indicate that overexpression of Smac promoted apoptosis through antagonizing the inhibition of XIAP of both distal and proximal events in the caspase cascade.

Cytosolic Smac bypasses the Bcl-2 inhibition. Since Bcl-2 may prevent Smac release from mitochondria, Smac function was analyzed in SHEP neuroblastoma cells transfected

with Bcl-2. Overexpression of Bcl-2 prevented the release of Smac and cytochrome c from mitochondria upon TRAIL treatment. Also, Bcl-2 inhibited activation of caspase-3 into active fragments and cleavage of the caspase-3 substrates PARP and DFF45. Interestingly however, Bcl-2 reduced, but did not prevent the initial cleavage of caspase-3 into the p24 intermediate fragment or cleavage of caspase-8 consistent with a block at the postmitochondrial level, e.g. by XIAP. It was investigated whether cytosolic Smac without the mitochondrial targeting sequence can bypass the Bcl-2 block. Ectopic expression of GFP-tagged Smac in the cytosol was controlled by fluorescence microscopy. Importantly, ectopic expression of cytosolic Smac sensitized SHEP neuroblastoma cells overexpressing Bcl-2 for apoptosis induction. Also, cytosolic Smac further enhanced treatment-induced apoptosis in SHEP vector control cells, consistent with high XIAP expression in these cells. Expression of cytosolic Smac per se showed no cytotoxic effect indicating that the release from IAP inhibition by Smac only becomes relevant upon apoptosis induction. The studies were further extended to different cell lines with Bcl-2 overexpression. Ectopic expression of cytosolic Smac sensitized Bcl-2 transfected glioblastoma (U87MG/Bcl-2, LN18/Bcl-2, LN229/Bcl-2) and breast carcinoma (MCF7/Bcl-2) cells for treatment with TRAIL, anti-CD95 antibody or doxorubicin. Thus, cytosolic Smac may bypass Bcl-2 inhibition in several cell types and in response to different pro-apoptotic stimuli.

In addition, overexpression of mitochondrial or cytosolic Smac sensitized neuroblastoma cells for gamma irradiation – induced apoptosis (see DRAWING). This indicated that Smac agonists, e.g. Smac peptides, may be used in combination with irradiation to enhance the effect of radiotherapy.

Smac peptides sensitize resistant tumor cells for death receptor or drug-induced apoptosis. The N-terminal 4 residues of Smac that are essential for inactivation of XIAP and thus for apoptosis induction, together with the 3 following residues, were linked to the protein transduction domain of the TAT protein to facilitate intracellular delivery (Smac peptide / PTD). Cellular uptake of Smac peptides was controlled by flow cytometry and fluorescence microscopy. Smac peptides markedly enhanced TRAIL-induced apoptosis and also sensitized for treatment with anti-CD95 antibody or cytotoxic drugs. Furthermore, Smac peptides sensitized several resistant cell lines with defects in apoptosis signaling for

treatment with TRAIL or doxorubicin, including neuroblastoma cells with Bcl-2 overexpression (SHEP/Bcl-2), neuroblastoma cells with absent caspase-8 expression (SH-SY5Y), melanoma cells with impaired Apaf-1 expression (Mel-HO) or pancreatic carcinoma cells with defective Ras/PI3 Kinase/Akt signaling (Panc-1).

- 5 To exclude that the observations were restricted to cell lines maintained in long-term culture, primary tumor cells derived from a malignant pleural effusion of a patient with neuroblastoma at tumor relapse with refractory disease were examined. Importantly, Smac peptides sensitized these patient's derived resistant neuroblastoma cells with high levels of XIAP and Bcl-2, for apoptosis induced *ex vivo* by TRAIL or anticancer drugs.

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- Smac peptides enhance the antitumor effect of TRAIL in glioblastoma *in vivo* and induce eradication of tumors.** The effect of Smac was examined in a glioblastoma tumor model *in vivo*. Glioma cells were implanted into the right striatum of athymic mice and Smac peptides and /or TRAIL were locally administered at day 7 and day 9 after tumor
15 inoculation. Importantly, Smac peptides significantly sensitized glioblastoma cells for TRAIL-induced apoptosis, while treatment with Smac peptides alone showed no antitumor effect. Complete eradication of preestablished glioblastoma tumors was only found in mice treated with the combination of Smac peptides and TRAIL in 33% (2 of 6) or 50% (3 of 6) of tumors. Combined administration of Smac peptides and TRAIL showed no acute or
20 delayed neurotoxicity as assessed by a compound neurological score, whereas 2 of 6 mice treated with TRAIL alone developed neurological deficits indicating that the combination of Smac peptides and TRAIL may also improve neurological outcome.

25 **MATERIALS AND METHODS**

Cell culture. Neuroblastoma (SHEP, SH-SY5Y), glioblastoma (U87MG, LN18, LN229), Panc-1 pancreatic carcinoma or MCF-7 breast carcinoma were maintained in RPMI 1640 medium (Life Technologies, Inc., Eggenstein, Germany) as previously described. 0.5×10^5

cells/ml were cultured in 24-well-plates for determination of apoptosis or in 75 cm² flasks (Falcon, Heidelberg, Germany) for protein isolation.

Determination of apoptosis. Cells were incubated with recombinant human TRAIL (PeproTech Inc., Rocky Hill, NJ), cisplatin (Sigma, Deisenhofen, Germany), doxorubicin (Amersham Pharmacia, Freiburg, Germany) VP-16 (Bristol Myers, Erlangen, Germany) or anti-CD95 (APO1) monoclonal antibody. Smac peptides corresponding to aa 56-62 were linked to the protein transduction domain of Tat protein (Interactiva GmbH, Ulm, Germany). For assessment of cellular uptake, FITC-labelled peptides were used. Quantification of DNA fragmentation was performed by fluorescence-activated cell-sorting (FACS) analysis of propidium iodide stained nuclei as previously described.

Western blot analysis and immunoprecipitation. Western blot analysis and immunoprecipitation were performed as previously described using mouse anti-caspase-8 monoclonal antibody C15 (1:10 dilution of hybridoma supernatant), mouse anti-caspase-3 monoclonal antibody (1:1000, Transduction Laboratories, Lexington, KY), rabbit anti-caspase-9 polyclonal antibody (1:1000, PharMingen, San Diego, CA), mouse anti-XIAP monoclonal antibody (1:1000, H62120, Transduction Laboratories), mouse anti-DFF45 monoclonal antibody (1:1000, Transduction Laboratories), rabbit anti-AIF polyclonal antibody (1:5000, kindly provided by G. Kroemer), rabbit anti-Smac polyclonal antibody (1:5000, kindly provided by X. Wang), mouse anti-COX4 monoclonal antibody (1:1000, Clontech Laboratories, Inc., Palo Alto, CA), mouse anti-Flag monoclonal antibody (1:1000, Sigma) or mouse anti- β -actin monoclonal antibody (1:5000, Sigma) followed by goat anti-mouse IgG or goat anti-rabbit IgG (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA). Enhanced chemiluminescence (ECL, Amersham Pharmacia) was used for detection. Expression of β -actin was used to control for equal gel loading.

Transfection experiments. SHEP neuroblastoma cells were transfected with expression plasmid pcDNA3.1 vector containing full length Smac cDNA or empty vector using lipofectamine transfection reagent (Life Technologies, Inc.) and cultured in 0.5 mg/ml

G418 (Life Technologies, Inc.). Transient transfections with pEGFPC1 vector containing GFP-tagged Smac without the mitochondrial targeting sequence (aa 1-55) ²⁶ were performed using gene porter transfection reagent.

5 *Preparation of mitochondria or cytosolic extracts.* Preparation of mitochondria or cytosolic extracts was performed using the ApoAlert cell fractionation kit (Clontech Laboratories) according to the manufacturer's instructions.

10 *Animal studies.* 5×10^4 U87MG human glioblastoma cells were stereotactically implanted into the right striatum of athymic mice (CD1 nu/nu, Charles River, Sulzfeld, Germany). At day 7 or at day 7 and day 9, mice were locally treated with Apo2L/TRAIL (2 μ g/4 μ l buffer) and/or Smac (1 mg/4 μ l buffer) or buffer only. Tumor cell volumes were measured at day 21 or 35 after tumor cell implantation as previously described. Neurological symptoms (alertness, behaviour, weight loss, focal neurological deficits) were evaluated daily and a compound score of all categories was formed (++: severe deficits, +: deficits, -: no relevant deficits). Statistical significance was assessed using ANOVA.

15 *Radiation experiments.* SHEP neuroblastoma cells transfected with vector control (white bars, FIGURE), mitochondrial Smac (hatched bars) or cytosolic Smac (black bars) were treated with 0.3-10 Gy gamma irradiation. Apoptosis was determined after 10 days by FACS analysis of propidium iodide-stained DNA. Mean and standard deviation of triplicates of a representative experiment are shown.